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A Salt-Inhibited Cytochrome *c* Reductase Obtained from the Moderately Halophilic Bacterium, *Micrococcus halodenitrificans*

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A membrane-bound cytochrome *c* reductase from *Micrococcus halodenitrificans* was inhibited by NaCl at concentrations as low as 0.05 M. The inhibition was competitive with respect to cytochrome *c* and noncompetitive with respect to reduced nicotinamide adenine dinucleotide. Thus the effect of NaCl was to increase the apparent K_m of the enzyme for cytochrome *c* and not to inhibit the intrinsic activity of the enzyme.

One explanation for the salt requirement exhibited by halophilic bacteria is based on the activation of their enzymes by relatively high concentrations of NaCl (7). A particulate reduced nicotinamide adenine dinucleotide (NADH) oxidase, obtained from the moderately halophilic bacterium *Micrococcus halodenitrificans*, was found to be maximally active at salt concentrations where cytochrome *c* reductase activity (which was also associated with the membrane fraction) was maximally inhibited. This inhibition was difficult to reconcile with the observation that cytochrome *c* reduction was a measure of the NADH dehydrogenase component of the NADH oxidase system, and that the internal salt concentration of *M. halodenitrificans* ($K^+ + Na^+$), when grown in medium containing 1 M NaCl, was reported to be approximately 0.6 M (2). This communication describes this seemingly anomalous observation, and suggests that it is a consequence of measuring velocity at a subsaturating concentration of cytochrome *c*.

M. halodenitrificans (ATCC 13511) was grown as previously described (4). Cells were harvested from cultures in the exponential phase, suspended in 1.0 M NaCl-0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid-hydrochloride buffer (*p*H 7.4) to give a buffer to cell ratio of 3, and disrupted at 15,000 psi in a French pressure cell operated at 4 C. The resulting suspension was centrifuged at 3,800 × *g* for 30 min at 4 C, and the turbid supernatant liquid was designated as the crude extract.

As shown in Fig. 1, crude extracts oxidized 0.006 μmole of NADH per min per mg of protein in the absence of added NaCl. As the concentration of NaCl increased, so did the rate of NADH oxidation, reaching a maximum value of 0.067 μmole of NADH oxidized per min per mg of protein in the presence of 0.6 M NaCl. NADH oxidase activity was inhibited at concentrations of NaCl greater than 0.6 M, so that in 3.0 M NaCl oxidase activity was approximately 60% of the maximum rate (inset, Fig. 1). When crude extracts were assayed for cytochrome *c* reductase activity, maximum reduction (0.91 μmole of cytochrome *c* reduced per min per mg protein) took place in the absence of added NaCl (Fig. 1). As the concentration of NaCl was increased, the rate of cytochrome *c* reduction rapidly decreased, with the result that, in the presence of 0.6 M NaCl, the rate of cytochrome *c* reductase activity was about 1% of the maximum rate. The sensitivity of cytochrome *c* reductase to NaCl was not related to the particulate nature of the enzyme since partial purification by high-speed centrifugation, deoxycholate solubilization of the resulting pellet fraction, and ammonium sulfate precipitation yielded an enzyme fraction as sensitive to NaCl as the the activity present in crude extracts (Fig. 1).

The report that cytochrome *c* formed complexes with phospholipids, which were disso-

ciated by salt (3), suggested that if the proteins from moderately halophilic bacteria were acidic, like the bulk proteins from the extremely halophilic bacteria (10), the binding of cytochrome c, a basic protein, to an acidic halophilic enzyme might be affected by the ionic strength of the medium. A consequence of this assumption was that the inhibition of cytochrome c reductase by NaCl should be competitive with respect to cytochrome c and noncompetitive with respect to NADH. As shown in Fig. 2, this was indeed the case. When the activity of the enzyme was determined at various concentrations of cytochrome c and NaCl (Fig. 2a), the effect of NaCl was to increase the slope but not the intercept of a double reciprocal plot. The effect of NaCl on the apparent K_m for cytochrome c was to change it from 0.011 mM in the absence of added NaCl to 0.073 mM in 0.05 M NaCl, 178 mM in 0.1 M NaCl, and 910 mM in the presence of 0.2 M NaCl. The observed inhibition of cytochrome c reductase activity at any concentration of NaCl was in good agreement with the inhibition to be expected from the apparent K_m at that concentration of salt and the concentration of cytochrome c present in the reaction mixture (0.16 mM). As shown in Fig. 2b, the inhibition of cytochrome c reductase activity was noncompetitive with respect to NADH.

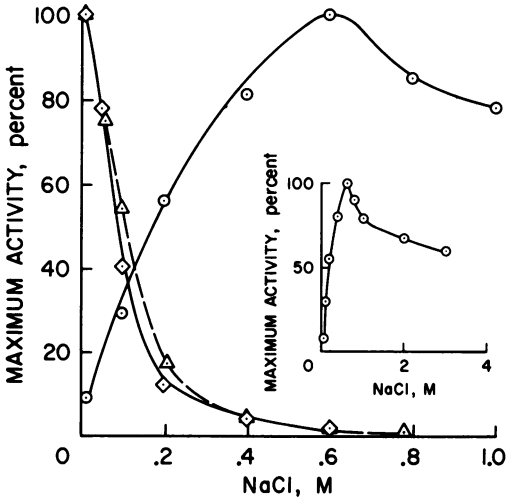

FIG. 1. *Effect of NaCl on NADH oxidase and cytochrome c reductase activities. NADH oxidase activity (⊙) was measured at 340 nm in reaction mixtures containing the following additions in a total volume of 1 ml: 100 μmoles of tris(hydroxymethyl) aminomethane (Tris)-hydrochloride (pH 7.4); 0.15 μmole of NADH; NaCl as indicated; and crude extract (405 μg of protein). The maximum specific activity, observed in 0.6 M NaCl, was 0.067 μmole of NADH oxidized per min per mg of protein. Cytochrome c reductase activity was measured at 550 nm in reaction mixtures containing the following additions in a total volume of 1 ml: 100 μmoles of Tris-hydrochloride (pH 8.8); 0.15 μmole of NADH; 160 μmoles of horse heart cytochrome c; and NaCl as indicated. ◇, Cytochrome c reductase activity in crude extracts; △, cytochrome c reductase activity in a partially purified and solubilized preparation. The maximum specific activities (micromoles of cytochrome c reduced per minute per milligram of protein) observed in the absence of added NaCl (10 μmoles of NaCl were carried over with the enzyme) were 0.91 and 2.93 in the case of the crude and partially purified extract, respectively. The millimolar absorptivities used for reduced cytochromes c and NADH were 19.6 mM^{-1} cm^{-1} (11) and 6.22 mM^{-1} cm^{-1} (5), respectively. In both assays, the extract was added to initiate the reaction which was carried out at room temperature (ca. 22 C). The results are corrected for any NADH oxidation which took place in the absence of the crude extract, or cytochrome c reduction which occurred in the absence of added NADH. No reoxidation of reduced cytochrome c was observed.*

The reduction of horse heart cytochrome c by extracts obtained from the moderately halophilic bacterium *Vibrio costicolus* and the nonhalophilic bacterium *Thiobacillus thioparus* was also inhibited by NaCl (Fig. 3). The inhibition of the enzyme obtained from *V. costicolus* was quantitatively similar to what was observed with the micrococcus enzyme and was also competitive with respect to cytochrome c. The enzyme from *T. thioparus* was also inhibited by NaCl although the inhibition was not as severe. As in the case of the enzymes from the moderately halophilic bacteria, this inhibition was also relieved by increasing the cytochrome c concentration. Cytochrome c reduction by extracts obtained from the extremely halophilic bacterium *Halobacterium cutirubrum* behaved in a different fashion (inset, Fig. 3). No activity was detected in the absence of added NaCl, and maximum activity was attained only when the salt concentration was at least 2.5 M. No inhibition was observed when the NaCl concentration was as high as 4 M. Whether this indicates that the binding of horse heart cytochrome c to the enzyme from *H. cutirubrum* did not involve electrostatic interactions, or that the forces binding cytochrome c were protected from the bulk medium, is not clear at the present time.

There are reports (1, 6) concerning the inhibition of certain enzymes obtained from moderately halophilic organisms that are inconsistent with their reported internal salt con-

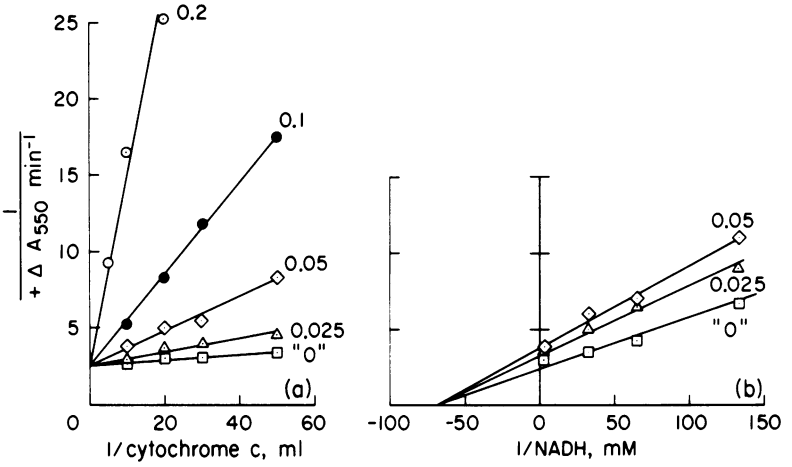

FIG. 2. *Inhibition of cytochrome c reductase activity by NaCl. Cytochrome c reductase activity was measured as described in the legend to Fig. 1 except that in (a) the concentration of cytochrome c was varied and in (b) the concentration of NADH was varied. In part (a), the desired concentration of cytochrome c was obtained from a stock solution that was 1.67 mM. The numbers adjacent to each line represent the molarity of NaCl present in the reaction mixture.*

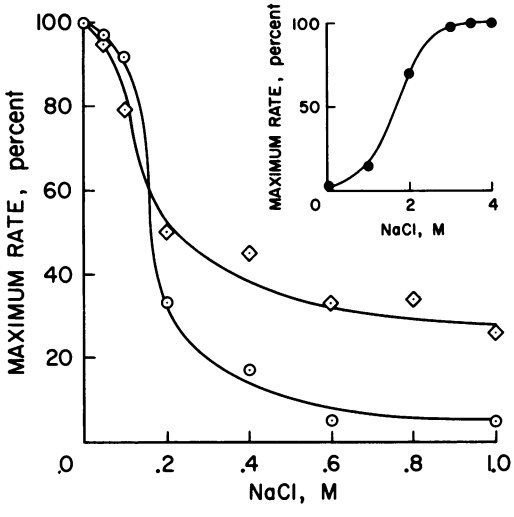

FIG. 3. *Inhibition of various cytochrome c reductase activities. Cytochrome c reductase activity was measured as described in Fig. 1. Crude extracts from T. thioparus (obtained from E. Johnson) were prepared as described by MacElroy et al. (8). The crude extract obtained from H. cutirubrum was prepared as described by Hochstein and Dalton (4). The maximum specific activities (micromoles of NADH oxidized per minute per milligram of protein) were 0.93 for V. costicolus (⊙), 0.54 for T. thioparus (◇), 0.19 for H. cutirubrum (●).*

centrations (2, 9). A similar situation exists in the case of certain enzymes found in extremely halophilic bacteria (7). Whether the intrinsic activities of these enzymes were indeed inhibited cannot be determined from the data since the enzymes were assayed at a single substrate concentration. In a related situation, an NADH dehydrogenase obtained from an extremely halophilic bacterium, while apparently activated by NaCl was, in fact, fully active in the absence of added salt (<10 mM NaCl) when the maximum velocity of the enzyme was determined (Hochstein and Dalton, *manuscript in preparation*). Whether these phenomena are widespread awaits further investigation.

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